Volume 15 Number 6 1987 Nucleic Acids Research

Mapping of DNA markers close to the fragile site on the human X chromosome at Xq27.3

M.Patterson, S.Kenwrick, S.Thibodeau[1], K.Faulk[1], M.G.Mattei[2], J.-F.Mattei[2] and K.E.Davies

Nuffield Department of Clinical Medicine, John Radcliffe Hospital, Oxford OX3 9DU, UK, [1]Department of Pathology, Children's Hospital, Denver, CO 80218, USA and [2]Centre de Genetique Medicale, INSERM U242, Hôpital de la Timone, 13385 Marseille, France

Received January 22, 1987; Revised and Accepted February 18, 1987

SUMMARY

We report the identification of a new RFLP detected by the DNA probe MN12, which is linked to both the fragile site on the X chromosome at Xq27.3 and the highly polymorphic locus detected by St14 (DXS52). In situ mapping confirms the localisation of MN12 distal to the fragile site. A detailed physical analysis of this region of the X chromosome using pulsed-field gel electrophoresis has shown that MN12, St14 and DX13 (DXS15) are physically linked within a region of 470kb. A long range restriction map around the MN12 locus reveals at least two candidate HTF islands, suggesting the existence of expressed sequences in this region.

INTRODUCTION

The fragile X syndrome is the most common form of X-linked mental retardation in man (for review see reference 1). The syndrome is associated with a fragile site at Xq27.3, which can be induced in cell culture by a variety of conditions such as reduced folic acid and thymidine concentrations (for review see reference 2). The fragile site is expressed only in a proportion of cells which ranges from less than 1% to 80% in affected males. Fragile X expression is also observed in affected females, although usually to a lesser extent than in males. There is however no simple relationship between the degree of fragile site expression and the severity of the mental impairment. At present, then, the nature of the association between the fragile site and the syndrome is elusive.

Although the fragile X syndrome was originally thought to be inherited as a classical, recessive X-linked mutation, there is now no simple model to account for the many unusual features of the transmission of the syndrome. For example, approximately one third of female carriers of the fragile X mutation manifest some degree of mental impairment, implying that the mutation cannot be regarded as recessive. Furthermore, many phenotypically normal males, termed transmitting males, have been identified who transmit the fragile X mutation to their daughters (3,4). Thus the mutation is not

fully penetrant in males. Finally, it appears that new mutations causing the syndrome occur exclusively in sperm.

The genetic analysis of the fragile X syndrome has been greatly improved by the use of DNA probes detecting restriction fragment length polymorphisms (RFLPs) localised around Xq27. This has led to the construction of a genetic map of the region (5,6). It is clear, however, that levels of recombination are relatively high, as expected for a region localised towards the telomere (7) and hence further RFLP markers are required for a more complete and detailed map. The genetic map is also complicated by the evidence for genetic heterogeneity found amongst affected families (8).

The various questions posed by the fragile X syndrome will begin to be answered only when a detailed physical characterisation of the region around Xq27.3 has been achieved. Towards this end, recently developed techniques such as pulsed-field gel electrophoresis (PFGE) (9,10) and the construction of chromosome jumping and linking libraries (for review see reference 11) should prove to be powerful tools. PFGE has been used in conjunction with infrequently cutting restriction enzymes for the long range physical analyses of various mammalian genomic regions including the Duchenne muscular dystrophy locus at Xp21 on the human X chromosome (12,13,14). We report here, firstly the identification and localisation of a new RFLP marker linked to the fragile site, on the distal side. Secondly, we present a long-range restriction map constructed by the use of PFGE, of the genomic region around the marker. Finally, we provide evidence for the relatively close physical linkage between this marker and two others (St14 and DX13) which are localised to the same region of the X chromosome (15).

MATERIALS AND METHODS

DNA probes

MN12 was originally isolated from an X-chromosome-enriched DNA library (16). The probe is a 2.8kb Eco RI/Msp I subfragment of a 3.8kb Eco RI fragment cloned in pUC8.

The St14 probe for locus DXS52 (Xq28) is a 3.0kb Eco RI fragment cloned in pBR322 which detects a multiallelic Taq I polymorphism (17). Probe DX13-7, a 2.0kb Eco RI fragment cloned in pAT-X detects a Bgl II polymorphism at the locus DXS15 (Xq28) (5).

Conventional Southern analysis

DNA isolation, restriction enzyme digestions, agarose gel

electrophoresis, Southern blotting and hybridisations were performed as described previously (18).

In situ hybridisation

Chromosome spreads were obtained from phytohaemagglutinin-stimulated lymphocytes from a girl with fragile X mental retardation, cultured for 96 hours in folate deficient medium. 5-Bromodeoxyuridine (5 BrdU) was added for the final seven hours of culture (60 μg/ml of medium) to ensure a post-hybridisation chromosomal banding of good quality.

MN12 plasmid DNA was tritium-labelled by nick translation to a specific activity of 5 X 10^7 dpm/μg. The radiolabelled probe was hybridised to metaphase spreads at final concentration of 500 ng/ml of hybridisation solution, as previously described (19).

Slides were covered with KODAK NTB_2 track emulsion and exposed for 10 days at 4°C. After development, the chromosome spreads were first stained with buffered giemsa solution and metaphases were photographed. R-banding was then performed by the fluorochrome-photolysis giemsa (F.P.G.) method, and metaphases rephotographed prior to analysis.

Pulsed-field gel electrophoresis techniques

Preparation of high molecular-weight DNA in agarose blocks, restriction enzyme digestion, electrophoresis, the preparation and use of yeast chromosomes and λ concatamers as markers, blotting, hybridisation and washing of filters and finally autoradiography have been described in detail previously (14). The cells used for this analysis were lymphoblastoid cell lines derived from normal males and females and in some cases, males affected by the fragile X syndrome.

RESULTS

The identification of an RFLP linked to the fragile site at Xq27.3

MN12 has previously been localised on the X chromosome to the region Xq28-Xqter using somatic cell hybrids (20). We were therefore interested in identifying an RFLP for this probe which might be useful for linkage analysis of the fragile X syndrome. Figure 1A shows a Bgl I RFLP detected by MN12, for which the frequency of the rare allele (10kb) is about 8%. This was determined from a panel containing 26 independent X chromosomes.

The Mendelian inheritance of the MN12 polymorphism is illustrated in figure 1B in a family which is also informative for the St14 probe (DXS52) and is affected by the fragile X syndrome. The clinical details of this family are presented elsewhere (21). The genotype of the affected boys

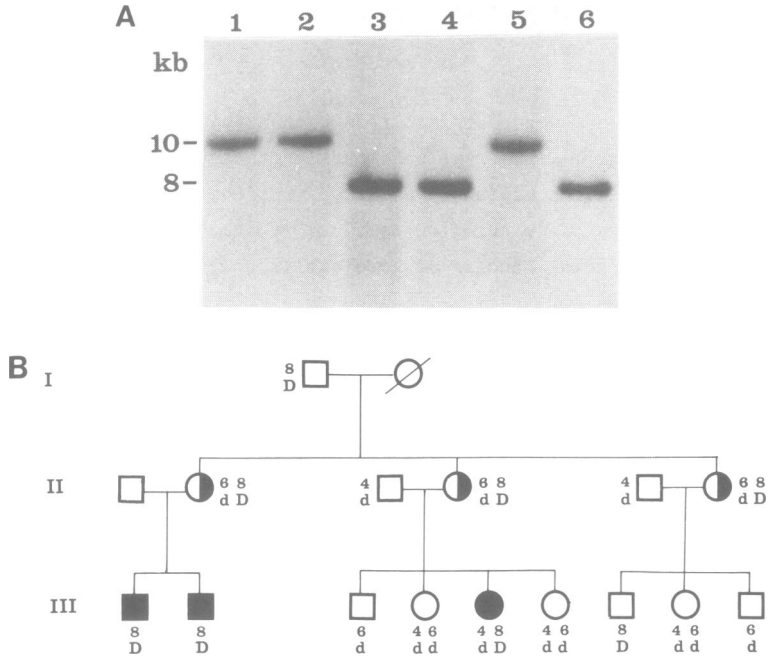

Figure 1. A: Hybridisation of MN12 to Bgl I digested DNA from six normal, males. MN12 detects two alleles at 10 and 8kb. B: A fragile X family informative for St14 and MN12. The St14 genotype is indicated by the numbers 4, 6 and 8 above the MN12 genotype denoted by d or D. Symbols should be interpreted as follows: ○ , □ normal female, male; ● , ■ affected female, male (affected individuals express the fragile site at Xq27.3 in greater than 10% of cells tested, and exhibit mental retardation characteristic of the fragile X syndrome (21)); ◑ carrier female. It should be noted that the female III.8 expressed the fragile site at Xq27.3 in 1% of her lymphocytes. Given this low level of expression and the lack of any form of mental impairment, we have, for the purposes of this work, assigned III.8 as unaffected. In addition, the fragile X status of individuals III.7 and III.9 has not been formally evaluated. A more detailed description of this family is provided in reference 21.

(III.1, III.2) is the same as that of the grandfather (I.1), whereas, with one exception (III.7), the genotype of the unaffected individuals indicates that they have not inherited the grandpaternal X chromosome. It is likely, therefore, that the original mutation was transmitted via the phenotypically-normal male (I.1) to his three daughters who would consequently be carriers as shown. In all individuals the MN12 and St14 loci cosegregate, including the normal male (III.7) who represents a presumptive crossover between these loci and the fragile X mutation. Similarly, in other families informative for MN12 and St14, recombination between these probe loci has not been observed (Steve Thibodeau and Huw Dorkins, personal communication). Thus, the genetic data suggest that MN12

is localised in the vicinity of St14, which is distal to the fragile site at Xq27.3 (15).

Localisation of MN12 by in situ hybridisation

In situ hybridisation was used as a direct means to determine the localisation of MN12 with respect to the fragile site at Xq27.3. Figure 2A shows examples of partial metaphases containing a fragile X chromosome which were hybridised to MN12, and in all cases silver grains are observed on the distal side of the fragile site. The distribution of 24 silver grains is summarised schematically in figure 2B, which provides strong evidence that MN12 is localised distal to the fragile site.

Physical mapping by PFGE

Figure 3A shows a long range restriction map covering about 420kb that was constructed using the probe MN12. In general, sites were mapped by double digestion using Sfi I in combination with another enzyme and sample autoradiographs are presented in figures 3B and 3C. It should be noted that this rationale allows for the mapping only of the site for a particular enzyme which is closest to the Sfi I site nearest to MN12. However, in cases where one of the enzymes in the double digest cleaves the DNA partially, it is possible to map more than one site for that enzyme. Two sites for Nru I were thus mapped within the 320kb Sfi I fragment containing MN12. An interesting feature of the map is the cluster of sites for the enzymes Bss HII, Nar I, Mlu I and Sac II, which must lie within 45kb of MN12. Evidence which allowed the mapping of the Bss HII and Mlu I sites in this cluster is presented in figures 3B and 3C. It is clear that both enzymes when combined with Sfi I in a double digest produce fragments of a very similar size. Thus sites for Mlu I and Bss HII are mapped close to each other at about 45kb from the Sfi I site nearest to MN12. Similar results allowed the mapping of Nar I and Sac II sites to the same region (figure 3A). Clusters of sites for the infrequently-cutting restriction enzymes used in these analyses must identify genomic regions which are rich in non-methylated CpG dinucleotides. Such regions which have been termed HTF islands (22) have been found in association with many genes (for review see reference 23).

In addition to this cluster, evidence for a second cluster of sites for the enzymes Sac II, Bss HII and Mlu I was found adjacent to the Sfi I site closest to MN12. However, the Bss HII and Mlu I sites were cleaved only in some samples. For example, in neither of the autoradiographs shown in figures 3B and 3C is there any evidence of an Mlu I or Bss HII fragment

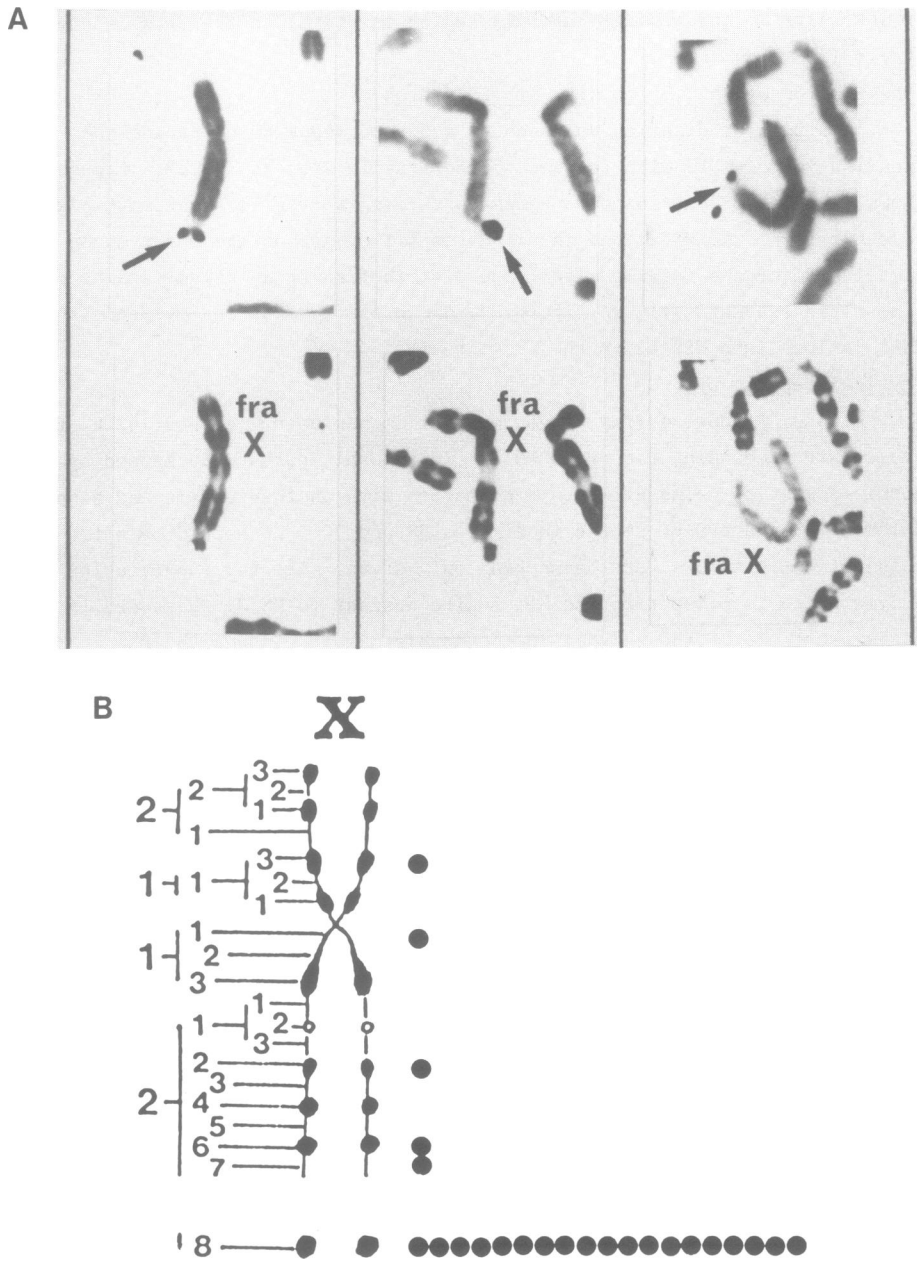

Figure 2. A: Partial metaphases showing the specific site of hybridisation for the MN12 probe. Arrows indicate the silver grains. Below, the fragile X chromosome (fra X) is identified by R banding. B: Diagrammatic summary of the silver grain distribution on the fragile X chromosome.

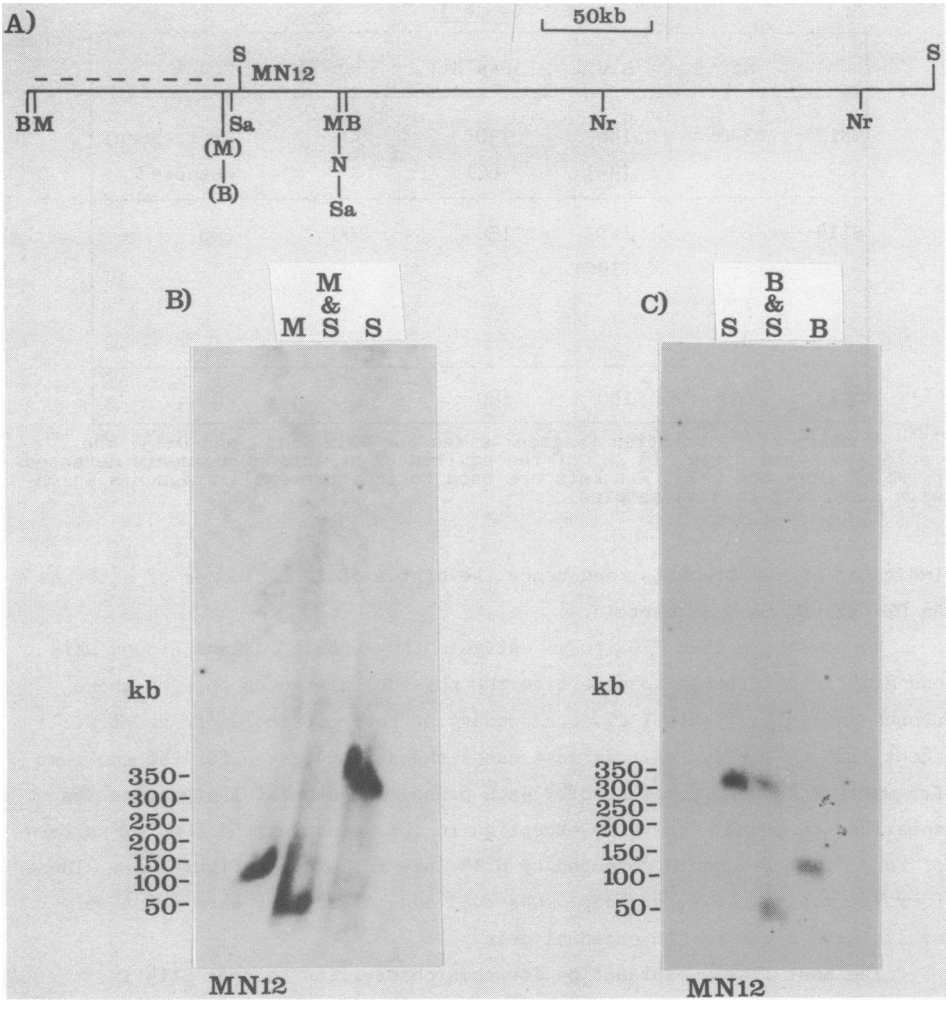

Figure 3. Long range restriction map around MN12. A: The dotted line indicates the region to which part of the St14 locus was localised as discussed in the text. Restriction sites are abbreviated as follows: B, Bss HII; M, Mlu I; N, Nar I; Nr, Nru I; S, Sfi I; Sa, Sac II. 3B, 3C: MN12 hybridisations used for the construction of the MN12 map. Normal male DNA was digested with the enzymes indicated at the top of each lane. Restriction enzymes are abbreviated as in figure 3A and the sizes and positions of relevant λ concatamers are indicated at the left.

detected by MN12 below 140 and 150kb respectively. However, in the partial Mlu I digest displayed in figure 5A, MN12 clearly hybridises to a fragment at 45kb. These and other similar results suggest that the Mlu I and Bss HII sites just to the left of MN12 in figure 3A are frequently methylated, as

Table 1

	Sfi I	Mlu I	Bss HII	Sac II	Nru I
MN12	320	140 (45)	150 (60)	50	No fragment detected
St14	60 25 11.5 8.5	140 (100)	150	100	200
DX13	60	180	180	100	200

Summary of restriction fragment sizes for MN12, St14 and DX13. The table shows the sizes, in kb, of the smallest restriction fragments detected by MN12, St14 and DX13. Brackets are used to indicate small fragments which were seen only in some samples.

indicated by the brackets, and hence the status of this cluster of sites as an HTF island is less certain.

We have also used PFGE to investigate the genomic regions around DX13 and St14 in an attempt to determine the physical distances between these probes and MN12. Table 1 gives a summary of the restriction fragment sizes identified by the probes. In most cases the size is given for the smallest fragment reproducibly detected by each probe, since partial digestion was sometimes observed. The only exception to this concerns the complex pattern of four Sfi I fragments detected by St14 (see for example figure 4A). These four fragments were reproducibly observed and, since they were relatively small, were sized on conventional gels.

The most likely explanation for this observation is that St14 is hybridising to several genomic sequences since it has been postulated that St14 is a member of a small family of related sequences (17). The original St14 probe hybridises to several Eco RI fragments all of which map to the Xq26-28 region and whose sizes add up to 65kb (17).

The observation that St14 hybridises to unique bands for Sac II (100kb) and Nru I (200kb) suggests that the genomic sequences which hybridise to St14 are clustered within a 100kb region. The existence of Sfi I sites within this region, however, would lead to the hybridisation of St14 to more than one fragment as seen in figure 4. St14 also hybridises to more than one fragment in Mlu I and Bss HII digests, suggesting the existence of sites for these enzymes within the genomic St14 region. However, it is difficult

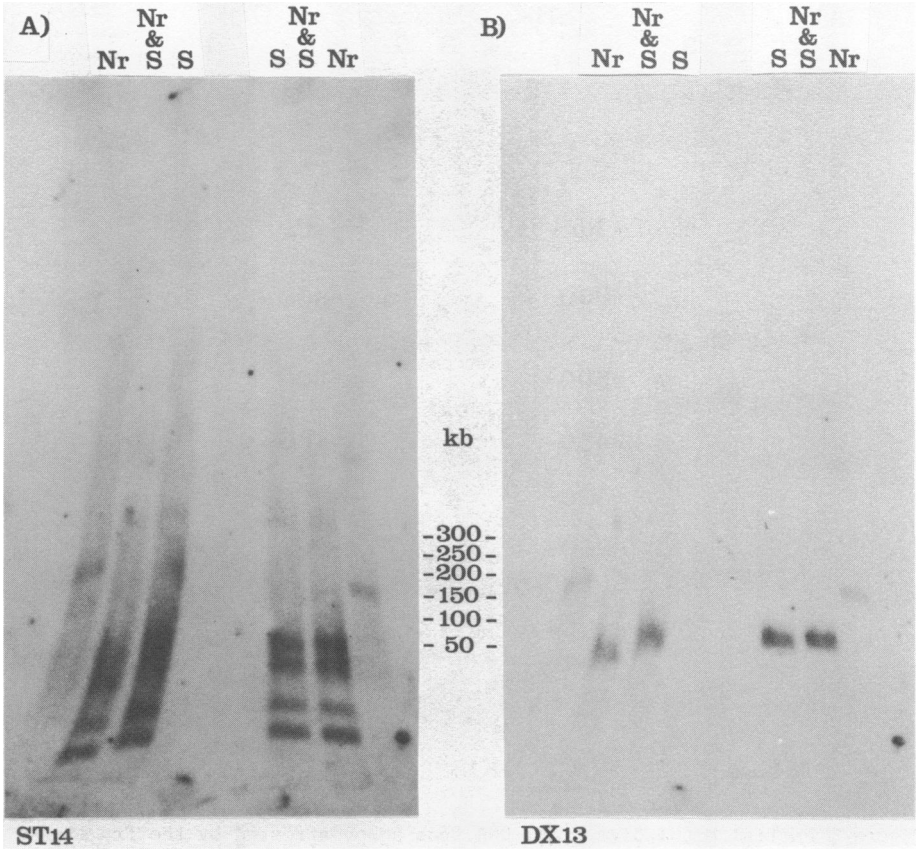

Figure 4 Physical linkage of St14 and DX13. A: Hybridisation of St14 to normal female DNA (the three left hand lanes) and normal male DNA (the three right hand lanes) digested as indicated.
St14 was eluted from the filter, which was then rehybridised to DX13 as shown in figure 4B. Sizes and positions of relevant λ concatamers are indicated, and restriction enzymes are abbreviated as in figure 3A.

to assess the contribution made to the hybridisation pattern by partial digestion which was observed particularly frequently with Mlu I (for example figure 5).

The sizes indicated in brackets in Table 1 refer to fragments which were detected by probes only in some digestions as discussed above.

Finally, Table 1 also provides evidence for the physical linkage of the three probes. Firstly, MN12 and St14 hybridise to the same-sized fragments for both Mlu I (140kb) and Bss HII (150kb) suggesting that the St14 locus lies within the region indicated by the dotted line in figure 3A. However,

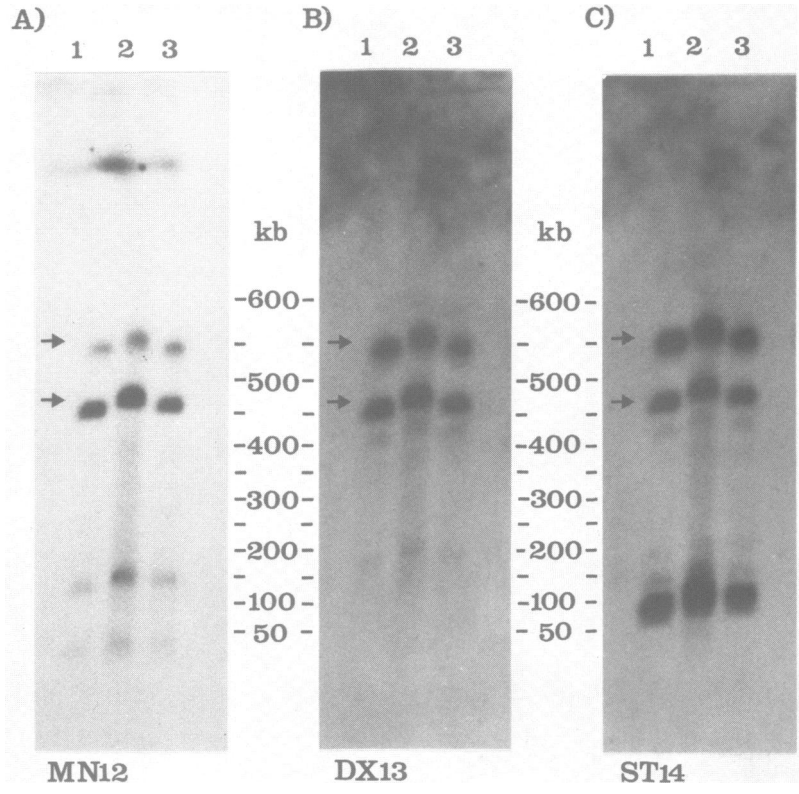

Figure 5 Partial Mlu I digestion. DNA from a boy affected by the fragile X syndrome was digested with varying amounts of Mlu I for 3 hours at 37°C: lane 1, 8 units; lane 2, 16 units; lane 3, 32 units. After PFGE and Southern transfer, the filter was hybridised to MN12 (A), DX13 (B) and St14 (C). In each case prolonged autoradiography for 6-7 days was carried out to show up any fainter bands. Arrows are used to indicate the 2 bands which hybridise to all three probes. The sizes and positions of relevant λ concatamers are indicated.

for the reasons discussed above, the St14 locus may contain sites for Mlu I and Bss HII and hence it is likely that the locus extends past the Bss HII and Mlu I sites at the left hand end of the map in figure 3A. Table 1 also indicates that DX13 and St14 share the same-sized fragments for Nru I (200kb) and Sac II (100kb) and furthermore the largest Sfi I fragment detected by St14 (60kb) is precisely the same size as the DX13 Sfi I fragment (figure 4). Double digestions using Sfi I and another enzyme have not, however, allowed the mapping of sites around St14 and DX13, since the double digestion gives bands identical to those in a single Sfi I digestion (see for example figure 4). Presumably, the relatively small Sfi I

fragments detected by DX13 and St14 are entirely contained within the larger fragment produced by the second enzyme. The results of a Bss HII/Sac II double digestion are incompatible with St14 and DX13 lying within a single Sac II fragment (results not shown).

The most conclusive evidence for the physical linkage of DX13, St14 and MN12 is provided by the partial Mlu I digests shown in figure 5. As indicated in the legend the three digestions were performed with varying amounts of enzyme from 8 to 32 units and incubated for 3 hours although the hybridisation pattern is virtually identical for each digestion. This suggests that in this case, the degree of partial digestion was determined by some property of the DNA sample such as its degree of methylation, rather than by the amount of enzyme. It should be noted that the DNA used in these partial digests derived from a boy affected by the fragile X syndrome. The hybridisation pattern observed with MN12 was initially thought to be different from that seen in normal controls suggesting that some sort of rearrangement was being detected in the affected boy. However, after relatively long exposures (7 days) of the filters, the results of which are displayed here, the normal MN12 band at 140kb can be seen faintly along with the infrequently observed 50kb band. Similarly, the partial digest fragments observed in the patient at 470 and 550kb appeared in more complete digests of normal males (results not shown). Thus the results in figure 5 in no way contradict those seen in normal male controls, and also demonstrate the need for caution in the interpretation of apparently altered bands in some samples.

It is clear from the hybridisation patterns displayed in figure 5 that all three probes identify 2 common partial bands at 470kb and 550kb (indicated by arrows) and that the maximum distance between the three probes is therefore 470kb. The pattern of smaller bands unique to each probe is also expected from the infrequent cleavage of the more proximal Mlu I sites.

Concerning the relative positions of DX13, St14 and MN12, we conclude from this physical analysis that St14 lies between DX13 and MN12, at a distance of less than 140kb from MN12 and possibly as little as 60kb from DX13.

DISCUSSION

The two polymorphic DNA probes, DX13 and St14 are localised approximately 10cM distal to the fragile site at Xq27.3 (15). We have localised a third probe (MN12) by genetic mapping and *in situ* hybridisation to the same region of the X chromosome.

We have shown that the three probes are physically linked within a region of less than 470kb and that St14 must lie between MN12 and DX13. The order of the three probes relative to the centromere of the X chromosome has yet to be established. To date, no crossovers have been detected between MN12 and St14 or DX13, although due to the relatively low frequency of the rare MN12 allele (8%), few families have been informative. However, crossovers have been observed between DX13 and St14 and the map distance between them can be estimated at 1-2cM (6,24). Since we have shown that the physical distance between the St14 and DX13 loci could be as little as 60kb, the general approximation that 1cM corresponds to about 1000kb no longer holds in this region of the X chromosome.

Linkage analysis of probes around the fragile site has not led to a consistent map of the region. For example, it has been suggested that the order of the probes St14 and DX13 may be reversed in different families (24). Physical mapping of the probes in these families would probably resolve such questions. As the data that we have generated is extended to incorporate other DNA markers from this region, it will be possible to establish the normal relative positions of these probes.

In general, the region analysed in this work has proved to be relatively rich in sites for infrequently-cutting restriction enzymes. Thus Xq27 appears similar to the class II region of the human major histocompatibility complex (25) but contrasts with the Xp21 region around the DMD locus (14). These observations suggest that Xq27 may be a highly expressed region. Interestingly, we have found evidence for two clusters of sites for the restriction enzymes we have used around MN12, and have argued that these clusters may correspond to HTF islands, which are often found at the 5' end of expressed sequences. However, it should be noted that the extent of an HTF island is about 1kb in general (23) and it is beyond the resolving power of our gels to claim that the clustered sites are within less than 5kb of each other. In view of the proximity of these clusters to MN12 it will be a relatively straightforward task to clone DNA around the clusters and investigate the nature of any associated expressed sequences.

ACKNOWLEDGEMENTS

We would like to thank the Medical Research Council and La C.N.A.M.T.S. for financial support. We are grateful to Huw Dorkins and Lynn Wilson for the communication of unpublished observations, to Jean-Louis Mandel for the use of the St14 probe, to Martyn Bell for technical

assistance, to Rachel Kitt for the preparation of this manuscript, and to Terry Smith and Sue Forrest for helpful discussions.

REFERENCES

1. Turner, G., and Jacobs, P.A. (1984) Adv. Hum. Genet. 13: 83-112.
2. Sutherland, G.R., and Hecht, F. (1985) Fragile Sites on Human Chromosomes, Oxford University Press, New York.
3. Sherman, S.L., Morton, N.E., Jacobs, P.A., and Turner, G. (1984) Ann. Hum. Genet. 48: 21-37.
4. Sherman, S.L., Jacobs, P.A., Morton, N.E., Froster-Iskenius, U., Howard-Peebles, P.N., Nielsen, K.B., Partington, M.W., Sutherland, G.R., Turner, G., and Watson, M. (1985) Hum. Genet. 69: 289-299.
5. Drayna, D., Davies, K.E., Hartley, D.A., Mandel, J-L., Camerino, G., Williamson, R., and White, R. (1984) Proc. Natl. Acad. Sci. USA 81: 2836-2839.
6. Drayna, D., and White, R. (1985) Science 230: 753-758.
7. Laurie, D.A., Hulten, M., and Jones, G.H. (1981) Cytogenet. Cell Genet., 31: 153-166.
8. Brown, W.T., Gross, A.C., Chan, C.B., and Jenkins, E.C. (1985) Hum. Genet. 71: 11-18.
9. Schwartz, D.A., and Cantor, C.R. (1984) Cell 37: 67-75.
10. Carle, G.F., and Olson, M.V. (1984) Nucleic Acids Res. 12: 5647-5664.
11. Poustka, A., and Lehrach, H. (1986) Trends in Genet. 2: 174-179.
12. Burmeister, M., and Lehrach, H. (1987) Nature 324: 582-585.
13. van Ommen, G.-J.B., Verkerk, J.M.H., Hofker, M.H., Monaco, A.P., Kunkel, L.M., Ray, P., Worton, R., Wieringa, B., Bakker, E., and Pearson, P.L. (1986) Cell 47: 499-504.
14. Kenwrick, S., Patterson, M.N., Speer, A., Fischbeck, K., and Davies, K. (1987) Cell in press.
15. Goodfellow, P.N., Davies, K.E., and Ropers, H-H. (1985) Cytogenet. Cell. Genet 40: 296-352.
16. Davies, K.E., Young, B.D., Elles, R.G., Hill, M.E., and Williamson, R. (1981) Nature 293: 374-376.
17. Oberle, I., Drayna, D., Camerino, G., White, R., and Mandel, J.-L.. (1985) Proc. Natl. Acad. Sci. USA 82: 2824-2828.
18. Davies, K.E., Pearson, P.L., Harper, P.S., Murray, J.M., O'Brien, T., Sarfarazi, M., and Williamson, R. (1983) Nucleic Acids Res. 11: 2303-2312.
19. Mattei, M.G., Philip, N., Passage, E., Moisan, J.P., Mandel, J.-L., and Mattei, J.F. (1985) Hum. Genet. 69: 268-271.
20. Wieacker, P., Davies, K.E., Cooke, H.J., Pearson, P.L., Williamson, R., Bhattacharya, S., Zimmer, J., and Ropers, H-H. (1984) Am. J. Hum. Genet. 36: 265-276.
21. Thibodeau, S., Dorkins, H.R., Faulk, K., Smith, A., Hagerman, R., Berry, R., King, A., and Davies, K.E. (1987) Submitted for publication.
22. Brown, W.R.A., and Bird, A.P. (1986) Nature 322: 477-481.
23. Bird, A.P. (1986) Nature 321: 209-213.
24. Brown, W.T., Jenkins, E.C., Gross, A.C., Chan, C.B., Krawczun, M.S., Duncan, C.J., Sklower, S.L, and Fisch, G.S. (1986) Hum. Genet., in press.
25. Hardy, D.A., Bell, J.I., Long, E.O., Lindsten, T., and McDevitt, H.O. (1986) Nature 323: 453-455.